AN NMR STUDY OF THE DYNAMICS OF INHIBITOR-INDUCED CONFORMATIONAL CHANGES IN LYSOZYME

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1. Introduction

The binding of the inhibitors *N*-acetyl glucosamine (GlcNAc), the dimer $(GlcNAc)_2$ and the trimer $(GlcNAc)_3$ to hen egg white lysozyme causes substantial changes in the chemical shift values of a number of resonances in the proton magnetic resonance (pmr) spectrum of the protein, [1,2]. These shifts undoubtedly result from conformational changes in the protein on inhibitor binding. Recently, many of the resonances in the lysozyme pmr spectrum have been assigned to specific protons of residues in the sequence, [2,3,4]. The region of protein involved in the conformational changes can therefore be defined. This paper not only defines the binding regions but also shows that protein pmr may be used to define the rates at which the protein conformational changes occur. Such data are compared to those from previous kinetic studies, thus showing which steps in a rate profile are protein conformational changes.

2. Experimental

Lysozyme from hen egg white was obtained from the Sigma Chemical Company, dialysed, and lyophilised before use. GlcNAc was obtained from BDH, and $(GlcNAc)_3$ was kindly donated by Dr L. N. Johnson (Molecular Biophysics, Oxford). Samples were dissolved in 99.8% D_2O, exchangeable hydrogens were replaced by deuterons by heating to 80°C at pH 4 for several minutes, and the pH was adjusted to 5.3 by using dilute NaOD and DCl. Protein concentrations of up to 8 mM were used.

Spectra were recorded using a 270 MHz Bruker spectrometer with an Oxford Instrument Company magnet and a Nicolet 1085 computer with a 293 pulse controller and a 294 disc system. Dioxan and acetone were used as internal standards. Other procedures were as described previously, [2–4]. Chemical shift values are quoted in parts per million (ppm) downfield from 2,2-dimethyl-2-sila-pentane-5-sulphonate. In the text values for 54°C and a measured pH value of 5.3 are quoted.

3. Results and discussion

3.1. *The spectra of the bound and unbound protein*

Addition of $(GlcNAc)_3$ to lysozyme causes changes in the protein pmr spectrum, until the $(GlcNAc)_3$ is in excess. Thereafter no further change in the spectrum takes place. This indicates tight 1:1 binding of the inhibitor to lysozyme, as expected. Comparison of the pmr spectrum of lysozyme in the bound form (excess $(GlcNAc)_3$) with the spectrum of the unbound protein shows that changes of two types have taken place.

3.1.2. Chemical shift values

The chemical shift values of certain resonances have altered. The largest shifts observed are of resonances assigned to the methyl groups of the Ile 98 (γ- and δ-) and of Met 105, to a γ-CH_2 proton of Ile 98 and to aromatic CH and NH protons of Trp 108, Trp 63 and Trp 62. These shift data will be fully reported separately (but see also [1]), and the following conclusions may be drawn. First, the shifts observed are all of groups close to the active site cleft of lysozyme. Secondly, all the shifts can be explained by changes in ring current shifts caused

by the movement of one or more of the aromatic residues Trp 62, 63 and 108, which could accompany inhibitor binding. Significant shifts of resonances of groups not close to these residues have not been observed. X-ray diffraction studies [5] and pmr studies of inhibitor resonances [6] have indicated that $(GlcNAc)_3$ binds in sites A, B and C. GlcNAc however binds only in, or close to, site C. The protein resonance shifts with GlcNAc bound are very similar to those with $(GlcNAc)_3$ bound. It thus is likely that the conformational change accompanying inhibitor binding is in the region of site C, i.e. close to Trp 62, 63 and 108 as deduced above. This observation would be similar to those of the X-ray diffraction studies which suggest that Trp 62 moves on inhibitor binding [5].

3.1.2. Linewidth

As well as changes in chemical shifts of protein resonances, there are changes in the linewidths of certain resonances. In particular, the resonances of the γ-CH_3 group assigned to Ile 98 and of a tryptophan C(2)H proton assigned to Trp 63 are broader than other resonances in the unbound protein. This broadening of these resonances has been observed to be dependent on temperature, being much reduced at temperatures over 60°C. We assign the cause of this broadening to be the existence of different local conformations, between which the rate of conformational change is only fast at high temperatures. At lower temperatures, exchange broadening takes place. However, in the presence of $(GlcNAc)_3$ or even GlcNAc, such that the protein is fully bound, the broadening does not occur even at low temperatures. It therefore appears that there is a change in the conformational mobility of groups in the binding site. The most likely situation is that the existence of different local conformations have been eliminated. In other words the protein conformation is better defined, or more restricted, in the presence of a bound inhibitor. This observation is again consistent with X-ray diffraction observations [5] where the conformation of Trp 62 is only well defined in the presence of a bound inhibitor.

Thus, both X-ray and pmr observations suggest that a local conformational change takes place on binding inhibitors to lysozyme. They also both suggest that the protein conformation is better defined in the presence of an inhibitor.

3.2. *Measurement of kinetic data*

Instead of examining spectra of the bound and unbound protein separately, the appearance of the spectra of solutions containing both bound and unbound protein can be examined. These spectra are affected by the rate of exchange between the two forms.

In fig.1, part of the spectrum of lysozyme at pH 5.3 and 37°C is shown as the concentration of $(GlcNAc)_3$ is increased. The resonance which is at 6.28 ppm in the spectrum of the unbound protein has been assigned [4,7] to Trp 63. Spectra corresponding to both the bound and unbound protein are superimposed, although some linebroadening is observed. In fig.2, similar spectra are shown for a resonance assigned to one of the γ-CH_2 protons of Ile 98 because this resonance is coupled to the resonance assigned [4] to the δ-CH_3 group of this residue. Again, superimposed spectra are obtained. In each case the difference in chemical shift between the resonance in the bound and unbound forms is 60 Hz. The observation of this slow exchange phenomenon shows that the rate of exchange between the two forms

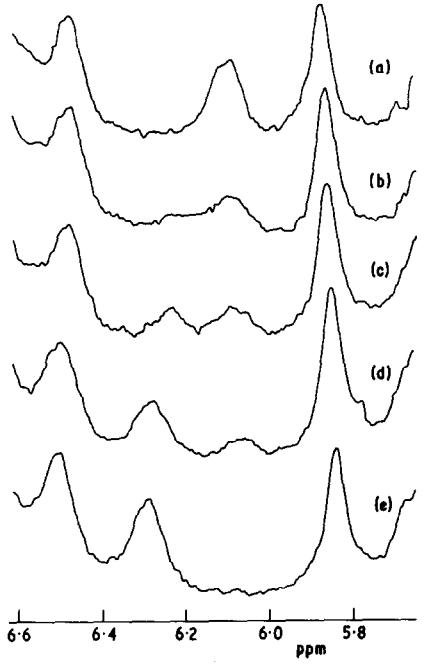

Fig.1. Part of the spectra of 8 mM hen lysozyme at 37°C, pH 5.3 in the presence of different concentrations of $(GlcNAc)_3$ which are (a) 7.7; (b) 5.1; (c) 4.2; (d) 1.9; (e) 0 mM. The central resonance is assigned to Trp 63.

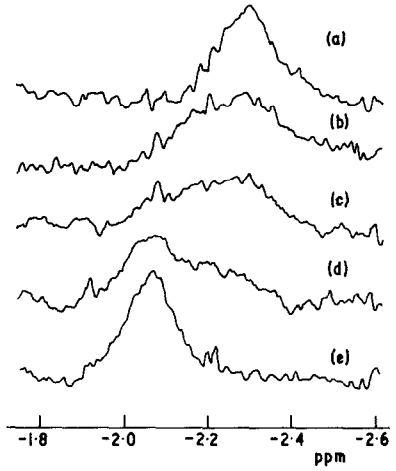

Fig.2. As fig.1 but showing the resonance assigned to a γ-CH_3 proton of Ile 98.

is small compared to this frequency separation.

In fig.3, spectra are shown corresponding to fig.1 but of a solution in which equal concentrations of bound and unbound protein are present. As the temperature is increased, the system passes from slow through intermediate to fast exchange. At the

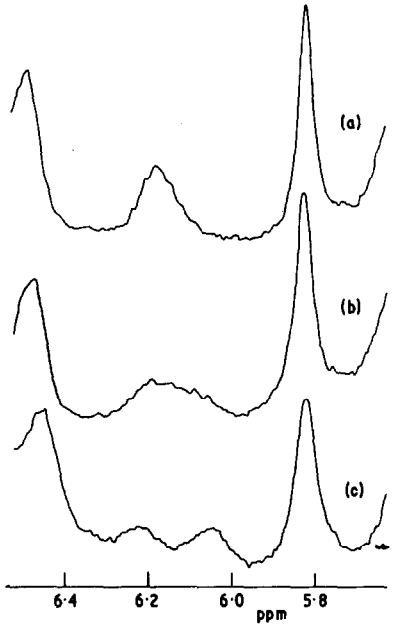

Fig.3. The effect of temperature on the spectrum of 8 mM hen lysozyme containing 4.1 mM $(GlcNAc)_3$ at pH 5.3. Temperatures are (a) 37°C; (b) 45°C; (c) 55°C.

temperature of coalescence of the two peaks (45 ± 3°C) the lifetime (τ_b) of the bound species can be estimated as 7.5×10^{-3} sec. Thus, for the first order rate process (bound conformation $\rightleftharpoons$ unbound conformation) a rate constant (k_{obs}) of 1.3×10^2 sec^{-1} at 45°C is obtained. At 25°C, k_{obs} is less than 20 sec^{-1}.

When the experiment was repeated with GlcNAc, fast exchange was observed even at 5°C. The lifetime of the bound species must be less than 10^{-3} sec, and k_{obs} greater than 10^3 sec^{-1} even at this low temperature.

Exchange effects on only two resonances have been discussed here. These resonances are well resolved and therefore easy to study. Similar effects on other resonances have also been observed but not yet analysed in detail.

3.3. *Interpretation of kinetic data*

The results described above on the fully bound and the partially bound protein permit direct interpretation of the kinetics of inhibitor binding to be made. Temperature jump and stopped-flow studies, [8] and pmr studies of inhibitor resonances, [9,10] have indicated that two steps are observed in the binding process of inhibitor (I) to lysozyme (E), that is

$$E + I \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} EI_1 \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} EI_2$$

The first step has been envisaged as a pre-equilibrium complex formation, the second as an isomerization process involving a rearrangement of the protein conformation. Now, our measured value of k_{obs} for $(GlcNAc)_3$ binding was clearly that for the conformational change which occurs on binding and results in shifts of specific resonances. This value is close to that obtained (under slightly different conditions) for k_{32} (10 sec^{-1} at pH 6.3 at 20°C) whilst k_{23} is faster (240 sec^{-1} at 20°C). k_{12} and k_{21} are of course much greater. For β-methyl-GlcNAc, $k_{23} \gg k_{32} > 10^3$ sec^{-1}, and similar values are expected for GlcNAc (both α and β anomers). In accord with these values, the conformational change is here observed to be rapid ($k_{obs} > 10^3$ sec^{-1} at 5°C). The present pmr results thus provide direct evidence that the observed rate constant k_{32} corresponds to a change in protein conformation in the region of site C of lysozyme. Note that it is unlikely that the complex EI_1 could be observed directly by pmr, first because its concen-

tration is very low and secondly because it will be in fast exchange with E.

Thus, protein pmr methods can be used to define the nature and rates of local conformational changes in proteins, by observation of resonances assigned to specific residues. In lysozyme these changes appear to be of two types. First, there are changes in the position of given groups and secondly in the mobility of these groups. The rate-controlling process could be important in the kinetics of substrate binding and reaction when the enzyme rate would be limited by binding steps remote from the attacking groups.

Acknowledgements

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